L Number	Hits	Search Text	DB	Time stamp
1	92	(bio$1film biofilm) and acid and (sds sodium near3 sulfate near3 sulphate)	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/21 15:01
2	2	(bio$1film biofilm) and acid and (sds sodium near3 (sulfate sulphate)) and sddd	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/21 10:56
3	19	(bio$1film biofilm) and acid and (sds sodium near3 (sulfate sulphate)) and salicylate	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/21 10:59
4	7155	(bio$1film biofilm) and acid and sds (sds sodium near3 (sulfate sulphate)) and salicylate	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/21 11:00
5	6	(bio$1film biofilm) and acid and sds and (sds sodium near3 (sulfate sulphate)) and salicylate	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/21 11:00
6	7	(bio$1film biofilm) near5 protein and acid and (sds sodium near3 sulfate near3 sulphate)	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/21 11:39
7	2	("5910420").PN.	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/21 13:34
8	0	"99/5578"	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/21 15:01
9	0	99/5578	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/21 15:01
10	367	"5578"	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/21 15:03
11	0	(99/51578).CCLS.	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/21 15:03
12	34	"51578"	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/21 15:04
13	1	"99/51578"	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/21 15:04
-	26626	BARBEAU GRAVEL HABI	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/18 17:21

-	282	BARBEAU.in. GRAVEL.in. HABI.in.	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/18 17:21
-	12	(BARBEAU.in. GRAVEL.in. HABI.in.) and (bio$1film biofilm)	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/18 17:22
-	8	(BARBEAU.in. GRAVEL.in. HABI.in.) and (bio$1film biofilm) and acid and (sds sodium near3 sulfate near3 sulphate)	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/01/18 17:35

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

By Anita Cheng

Purpose

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique is used to assess the purity and estimate the molecular weight of a protein (Horton et al., 2002).

Method

Polyacrylamide gel is polymerized from a solution of acrylamide monomers into crosslinking chains, forming a semisolid matrix of suspensions in water (Lodish et al., 2000). This matrix is buffered to weakly basic, so most proteins will be anionic and migrate to the anode of the gel when an electric current is applied. The rate at which the proteins move in the gel is affected by the electric current, the pore size of the gel, and the size of the protein (i.e. smaller proteins move faster in the gel) (Horton et al., 2002).

Sodium dodecyl sulfate (SDS) is an anionic detergent with a long hydrophobic tail that binds to the hydrophobic side chains of amino acids at a constant ratio of 1.4g of SDS to 1g of polypeptide, proportional to the molecular weight of the protein (Hames, 1998). Larger proteins bind to more SDS molecules. This ratio ensures that all SDS-protein complexes have a similar mass:charge ratio, eliminating the intrinsic charge of the protein as a factor affecting migration (Rybicki and Purves, 2001). SDS denatures the protein and dissociates multimeric proteins into its subunits, causing extended conformations in the polypeptides and thus eliminating the effects of the shape of the protein in migration (Lodish et al., 2000). This leaves protein size (mass) as the only factor affecting the separation of proteins in electrophoresis.

SDS is added to the gel and protein before and during electrophoresis, and is often accompanied by heating the protein at 100°C for a few minutes to denature the protein (Lodish et al., 2000). Reducing agents are used to reduce disulfide bonds (e.g. b-mercaptoethanol) (Hames, 1998). The proteins are often stained with a fluorescent dye (e.g. Bromophenol blue) before they are loaded into the wells of the gel and electrophoresed. Bands appear on the gel, showing the migration distances of different sized proteins. Migration distances are characteristic to specific proteins, and can be used to assess the purity of protein. If the protein of interest is unidentified, it can be compared to the migration distances of a known protein for an estimation of molecular weight.

Applications

SDS-PAGE, although primarily used to estimate protein size and assess protein purity, is also used to purify proteins. The separated proteins in respective bands of gel can be cut out, electroeluted into a buffer solution, and removed of salts, yielding a pure protein which can be used for structural analysis or antibody production (Horton et al., 2002). SDS-PAGE is used in many areas of discipline, such as biochemistry. For example, it was used to identify mannoproteins in the cell wall of velum-forming yeast, which is important in wine production (Alexandre et al., 2000). This technique is also widely used in forensic sciences. For example, it was used to analyze dentin proteins from human teeth, a reliable marker to for estimating age (Martin-de las Heras et al., 2000).

Limitations

SDS-PAGE becomes less effective when the proteins to be analyzed are of similar molecular weights, since the proteins will migrate anomalously, resulting in poor resolution (Hames, 1998). This technique cannot resolve proteins with molecular weights that are substantially less than the molecular weight of SDS because the SDS-protein complexes would all have approximately the same mass (i.e. mass of SDS) (Dunn, 1993). Since SDS binds to hydrophobic regions of amino acids, this method becomes ineffective if the proteins are richly hydrophilic and cannot bind efficiently to SDS (Hames, 1998). Proteins with non-proteinaceous components also do not bind efficiently to SDS (eg. Glycoproteins, lipoproteins) (Dunn, 1993). Proteins rich in acidic residues do not bind well to SDS either because they repel the negative charge on SDS. Highly basic residues may be analyzed to have an apparent molecular weight much greater than the actual molecular weight because basic amino acids are highly

attracted to the negative charge on SDS, resulting in the increase of the mass:charge ratio of the SDS-protein complex, thus lowering electrophoretic mobility (Hames, 1998). Also, proteins with high proline content may be perceived to have a higher apparent molecular weight because these rigid residues can change the conformation of the SDS-protein complex; this bulky shape slows migration (Hames, 1998).

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Rybicki, E., and Purves, M. June 2001. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE), http://www.uct.ac.za/microbiology/sdspage.html, accessed October 2001.

Helpful Resources

http://www.uct.ac.za/microbiology/sdspage.html This site provides a good and brief overview of the topic.

http://www.electrophoresis.apbiotech.com/aptrix/upp00919.nsf/Content/Elpho_1D_SDS+PAGE This website contains more details about the protocol and materials needed for SDS-PAGE.

Gel Electrophoresis of Proteins: A Practical Approach 3rd ed. This book dedicates an entire section to SDS-PAGE and contains well organized information about the theory behind this technique.

http://www.altcorp.com/SlideShows/Thimerosal/sld040.html This slide is an example of a SDS-PAGE gel, the bands of separated protein can be seen here.

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CAVEAT LECTOR

EXPERIMENTAL PROCEDURE FOR THE DETECTION OF A RARE HUMAN mRNA WITH THE DIG SYSTEM

Barbara Rueger, Julia Thalhammer, Irmgard Obermaier, and Stefanie Gruenewald-Janho

Boehringer Mannheim Customer Service Lab, International Support, Mannheim, Germany

2. MATERIALS AND METHODS

Electrophoresis conditions and sample preparation

See Table I for required solutions. Prepare all solutions with sterile dimethyldicarbonate-treated distilled water, then autoclave; we use dimethyldicarbonate instead of diethylpyrocarbonate because of DMDC's lower toxicity. When preparing Northern blots, use sterile solutions and sterile trays as much as possible. Treat gel boxes with ethanol prior to the run.

Table 1. Solutions required for Northern blots.

RNA loading buffer	Prepare fresh solution daily. 250 µl formamide, deionized 83 µl formaldehyde, 37% (w/v), final conc. 7.7% 50 µl 10X MOPS buffer 0.01% (w/v) bromophenol blue
10X MOPS	200 mM morpholinopropan-sulfonic acid 50 mM sodium acetate 10 mM EDTA pH 7.0 Make up in sterile H_20 and autoclave. After autoclaving, the solution will turn yellow. This does not interfere with the quality of the result, as has been occasionally believed.

20X SSC, sterile	3 M NaCl 300 mM sodium citrate, pH 7.0, autoclave
Low stringency wash buffer 1	2X SSC 0.1% sodium dodecyl sulfate (SDS), prepare a 10% stock solution, filter prior to use
High stringency wash buffer	0.1X SSC 0.1% SDS, prepare a 10% stock solution, filter prior to use
Washing buffer*	100 mM maleic acid, 150 mM NaCl, pH 7.5 (+20°C) 0.3%(v/v) Tween® 20
Maleic acid buffer*	100 mM maleic acid, 150 mM NaCl, pH 7.5 (+20°C)
Blocking solution*	1 % (w/v) Blocking reagent for nucleic acid hybridization dissolved in Maleic acid buffer and autoclaved. Blocking solution is cloudy and should not be filtered. It is stable for at least two weeks when stored at +4 °C, but must then be brought to room temperature before use.
Detection buffer	100 mM Tris-HCl 100 mM NACl, pH 9.5 (+20°C)
*Available in a ready-to-use form in the DIG Wash and Block Buffer Set (Cat. No. 1 585 762).	

1. Prepare a formaldehyde/agarose gel. We use MOPS-formaldehyde (2% final concentration) agarose gels exclusively and typically prepare them with 1-1.5% Agarose MP.

2. Prepare fresh formamide/formaldehyde RNA loading buffer each day. If not prepared fresh for each use, the formaldehyde will react with the formamide during storage. The bromophenol blue will turn from blue to green during storage and degrade the target.

3. Denature the RNA target sample for 10 min. at 65°C in RNA loading buffer, and place on ice immediately. For example, to 5 µl (100 ng, 50 ng, 5 ng) of target RNA, add 10 µl RNA loading buffer.

We do not use glyoxal denaturation of RNA because (i) glyoxal must

be freshly deionized prior to use for best results, and (ii) it has been reported that glyoxal may react with RNA to form complexes that are inhibitory to efficient transfer.

4. Load the dry gels, add electrophoresis buffer to the edges of the gel, allow the RNA to move into the gel at high voltage, and then add electrophoresis buffer to submerge the gel.

Note that our RNA loading buffer does not contain Ficoll or glycerol, which are generally used to simplify loading.

5. Run the gel with 1X MOPS buffer at 25 V overnight under a fume hood. Running gels overnight produces better separation. We include 1 mg/ml ethidium bromide in our running buffer to stain the RNA during the separation; ethidium bromide does not interfere with successful Northern blot hybridizations as has been occasionally reported. However, when running gels at high speed for a short time, we have sometimes observed that one half of the blot stains black after detection. This is probably a result of ethidium bromide running in the opposite direction of the RNA. In a short run, ethidium bromide does not pass through the total gel, and the "running front" becomes visible on the blot.

RNA transfer

1. Pre-equilibrate the gel in sterile 20X SSC for 10 min. This removes any remaining formaldehyde.

2. Transfer the RNA to a nylon membrane, using sterile 20X SSC as transfer buffer. To obtain the highest possible sensitivity, a positively charged nylon membrane is required. We always use the positively charged nylon membrane from Boehringer Mannheim, which has been specially developed to give the highest sensitivity with the DIG System. It is tested for a lower and an upper limit of charge, which is extremely important when working with nonradioactive probes at the high probe concentrations required for optimal results. Each lot is also quality controlled with DIG-labeled probes in a dot blot detection. Uncharged membranes can be used if highest sensitivity is not required.

3. Fix the nucleic acids to the membrane. In our labs, we have almost exclusively switched to UV-cross linking (at 120 mJ) because of the speed and convenience (*i.e.*, with the Stratalinker auto-program). However, baking for 30 min at 120°C has proved equally efficient with the Boehringer Mannheim nylon membrane. When baking the membrane, wash it in 2X SSC or sterile water to remove the 20X SSC prior to baking.

4. After UV cross-linking, briefly wash the membrane in water, and air dry it. This removes residual 20X SSC, which is essential prior to

hybridization. Alternatively, you may briefly wash the membrane in 2X SSC or sterile distilled water immediately after transfer (*i.e.*, prior to cross linking); however, this produces bands that are not as sharp.

Probe labeling

The ß-actin RNA is available already DIG-labeled (Cat. No. 1 498 045). For the CTF1 probe, we used the labeling procedure summarized here:

1. Linearize 1 µg template DNA with a restriction enzyme that generates 5' overhanging ends (1).

2. Treat linearized template DNA with phenol (2), ethanol precipitate.

3. Perform *in vitro* transcription with T7 RNA polymerase according to the pack insert of the DIG RNA Labeling Kit.

4. Evaluate the efficiency of labeling reaction by a standard direct detection as described in The DIG System User's Guide for Filter Hybridization (3).

We recommend a final labeled probe concentration of 100 ng/ml hybridization solution.

Prehybridization, hybridization, and stringency washes

1. In a sealed plastic bag, prehybridize the blot in 25 ml hybridization solution (lacking the probe) for 30 min at 68°C. We use DIG Easy Hyb buffer for all blot applications. This buffer has been specially developed for nonradioactive probes and has many advantages. It is quality controlled in a Southern blot and a Northern blot hybridization and has been evaluated for all blot applications. It contains urea instead of formamide, so it is not toxic. Hybridization conditions corresponding to the presence of 50% formamide are applied.

The hybridization temperature of 68°C has been empirically identified to be optimal for most RNA:RNA hybrids in the presence of 50% formamide. In rare cases when the prospective hybrid will have very high G+C content, this temperature may be increased.

2. Denature the probe for 5 min at 100°C, then immediately chill on ice.

3. Prewarm 3 ml (calculated for 67 cm^2) hybridization buffer to 68°C, then add the probe to a concentration of 100 ng/ml.

4. Hybridize the blot at 68°C overnight in a sealed plastic bag.

5. Remove the blots carefully from the bag. Immediately wash them

twice, 5 min per wash, with shaking in low stringency wash buffer 1 (2X SSC, 0.1% SDS) at room temperature.

During the washes, shake the tray, and use excess amounts of buffer to prevent the membranes from sticking to the tray and partially drying. Never allow the blots to dry between prehybridization and the final washes.

6. Wash the blots twice, 15 min per wash, with shaking in prewarmed high stringency wash buffer 2 (0. lX SSC, 0.1% SDS) at 68°C.

It is extremely important to prewarm the washing buffer for these stringency washes.

After the stringency wash, the blot may be stored dry at 4°C (if the blot will not be stripped and reprobed) or placed in wash buffer for immediate immunological detection.

Chemiluminescent detection

1. Centrifuge the vial of antibody conjugate in a microcentrifuge for 5-10 min to pellet precipitates that form during storage. Use antibody conjugate from the surface of the solution only.

If not removed, the precipitates will lead to dark spots on the membrane during chemiluminescent detection. The antibody can be stored again at 4°C, but this centrifugation procedure must be repeated prior to the next use.

2. Perform chemiluminescent detection according to the pack insert, or apply the "Transparency technique". See Table 2 for a summary of both methods of applying the chemiluminescent substrate. We used the following "Transparency Technique" (steps 3-5 below) to apply the chemiluminescent substrate in our examples.

3. Place the membrane on a transparency. Add approximately 500 µl of diluted chemiluminescent substrate per 100 cm^2 membrane.

4. Cover the membrane with a second transparency sheet, and incubate for 5 min at room temperature.

5. Allow excess liquid to drip off, and seal the "sandwich."

6. If CSPD® chemiluminescent substrate was used, preincubate the membrane for 10 min at 37°C.

This activates the decay of the unstable intermediate formed upon turnover of the substrate by alkaline phosphatase. If CDP~Star™ substrate was used, omit this step.

7. Place the "sandwich" in an X-ray cassette, and expose to X-ray film.

When using the CSPD substrate, exposures may also be performed at 37°C to obtain a faster result.

Table 2. Experimental procedure for detection of rare human mRNA on Northern blots.

Electrophoresis conditions and sample preparation	
Gel type	1.5% MOPS-formaldehyde (2%) agarose gel
Running buffer	1X MOPS
Running conditions	25 V overnight
RNA loading buffer	Formamide/formaldehyde
Sample denaturation	10 min at 65°C; place on ice immediately
RNA transfer	
Membrane	Boehringer Mannheim's Nylon membrane, positively charged or uncharged membrane
Gel equilibration	10 min in sterile 20X SSC prior to transfer; remove formaldehyde
Transfer buffer	sterile 20X SSC
Fixation	UV-cross-linking at 120 mJ; wash membrane briefly in water; air dry
Probe labeling	
RNA probe	Linearize template DNA, phenolize, transcribe *in vitro*
Probe concentration and preparation	
Denature probe for 5 min at 100°C. Chill on ice immediately. Add DIG-labeled RNA to prewarmed DIG Easy Hyb to 100 ng probe/ml; this solution may be filtered through 0.45 mm cellulose acetate filter (Schleicher & Schuell, FP-03012) if desired.	
Hybridization	
Pre hybridization	30 min at 68°C in 25 ml DIG Easy Hyb in a sealed plastic bag
Hybridization buffer	3 ml (67 cm^2 membrane) DIG Easy Hyb
Hybridization	68°C (for RNA:RNA hybrids) overnight in sealed plastic bag or roller tubes
2 washes	Low stringency wash buffer 1(2 X SSC, 0.1% SDS), 2 x 5 min at room temperature; shake
2 washes	Prewarmed (68°C) high stringency wash buffer 2 (0.1 X SSC, 0,1% SDS), 2 x 15 min 68°C; shake

Possible stopping point	Transfer membrane to wash buffer for detection, or air-dry and store at 4°C

Chemiluminescent detection

Antibody conjugate	Centrifuge 5-10 min; take from surface only
Equilibration	5 min washing buffer
Blocking	30 min in 1% blocking buffer
Antibody reaction	30 min with antibody diluted 1:10000 1% in blocking buffer
2 washes	2x15 min in wash buffer
Equilibration	2 min in Detection buffer
Alkaline phosphatase reaction	Dilute CSPD substrate 1:100 in detection buffer

Chemiluminescent detection (Choose from two substrate application methods)

1. Dipping technique

1. Incubate membrane shortly in 10 ml of diluted chemiluminescent substrate.

2. Briefly dry on Whatman 3MM paper.

Seal damp, and expose to X-ray film.

3. Diluted chemiluminescent substrate can be stored at 4°C after filtration and addition of 0.1 mM sodium azide; these precautions are necessary to avoid bacterial contamination.

2. Transparency technique

1. Place membrane on transparency.

2. Add approx. 500 µl/100 cm2 membrane.

3. Cover with second sheet of transparency, and incubate for 5 min.

4. Let excess liquid drip off and seal.

5. Place in X-ray cassette, and expose to film.
